The metabolic enzyme locus *Triosephosphate Isomerase* (*Tpi*) in *Drosophila melanogaster* is sensitive to the pairing- dependent *trans* interaction transvection

By

Patrick O'Donnell

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The Faculty of Science, Engineering and Architecture
Laurentian University
Sudbury, Ontario, Canada

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Thesis Examiners/Examinateurs de thèse:

Dr. Thomas Merritt
(Supervisor/Directeur(trice) de thèse)

Dr. Eric Gauthier
(Committee member/Membre du comité)

Dr. Céline-Larivière
(Committee member/Membre du comité)

Dr. Michele Longworth
(External Examiner/Examinateur externe)

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. David Lesbarrères
Monsieur David Lesbarrères
Acting Dean, Faculty of Graduate Studies
Doyen intérimaire, Faculté des études supérieures

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Abstract

Recent advances in our ability to visualize and quantify interactions between chromosomes have made the study of these interactions a key step in advancing our understanding of gene regulation. A special form of *trans* interactions, called transvection, occurs when homologous chromosomes are physically paired in somatic cells. I have characterized interactions at the *Triosephosphate isomerase* (*Tpi*) locus, present on the third chromosome, in *Drosophila melanogaster* using a combination of enzymatic and transcriptional assays and a series of low activity *Tpi* alleles I generated. I found significant interactions in *trans* at *Tpi* which showed a dependence on pairing of homologous chromosomes, this dependence classifies the *trans* effects at *Tpi* as the special form of regulation, transvection. These transvection effects at *Tpi* are also sensitive to genetic background, where the excision alleles have different transvection effect interactions based on the variation of the third chromosome with which they are paired. The presence of transvection at *Tpi* provides a new locus at which to study chromosomal interactions and confirms previous results that pairing dependent interactions, *in vivo*, are subject to a number of complex regulatory elements.

Keywords

Gene regulation, transvection, *trans*-interactions, Triose phosphate isomerase, Tpi, Drosophila melanogaster

Co-Authorship Statement

Chapter 1 is an introduction to the larger concepts of chromosomal architecture and organization in the nucleus, as well as a general introduction to interactions in *cis* and *trans* with an emphasis on transvection. Thomas J.S. Merritt (TJSM) provided valuable editorial feedback as well as input on the organization of ideas.

Chapter 2 is written in the form of a manuscript for submission to the journal *Biochemical Genetics*. I am the first author with co-authors Xinyang (David) Bing (XB) and TJSM. XB provided two of the excision alleles used in the experiments published here. TJSM contributed to the conception of the study as well as providing troubleshooting advice for assays.

Chapter 3 is a summary of the general conclusions including a possible model for transvection at *Tpi*. Suggestions for further experiments aimed at investigating more fully the interactions between homologous chromosomes both at *Tpi* and across chromosome arms have also been included.

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List of Abbreviations

3C: Chromatin conformation capture

4C: Circularized chromatin conformation capture

BDSC: Bloomington drosophila stock center

BEAF-32: Boundary element-associated factor of 32kD

Bx: *Beadex*

BX-C: Bithorax complex

Cas9: CRISPR associated protein 9

CRISPR: Clustered, regularly interspaced, short palindromic repeat

Ct: Threshold cycle

CTCF: CCCTC-binding factor

CP190: Centrosome-associated zinc finger protein 190

DHAP: Dihydroxyacetone phosphate

EMS: Ethyl methanesulfonate

FISH: Fluorescence in situ hybridization

FRT: FLP recombination target

G3P: Glyceraldehyde-3-phosphate

gRNA: guide RNA

HSD: Honestly significant difference

LCR: Locus control region

Men: *Malic enzyme*

RT-qPCR: Real time quantitative polymerase chain reaction

RS: Rearrangement screen

Su(Hw): *Suppressor of hairy wing*

Tpi: *Triosephosphate isomerase*

Ubx: *Ultrabithorax*

z: *zeste*

Chapter 1 Genome organization, interactions in *cis* and *trans* and transvection.

1.1 Project outline

An understanding of the relationship between genomic architecture and functionality is an integral step in the development of a more thorough model of transcriptional regulation. Perhaps one of the largest barriers to a consistent predictable relationship between genomic architecture and gene expression is the variability of phenotypes across organisms despite their having identical genetic sequences. Our current understanding of phenotypic plasticity has moved toward implicating a variety of factors as modulators of genetic mechanisms, which consequently control changes in phenotype (Reviewed in Schlichting and Smith 2002). The importance of genetic regulation on phenotype leads to a need to improve our understanding of how genes are regulated. It is well known that genes can be controlled by regulatory elements in *cis* (along a chromosome) and *trans* (between chromosomes) and variation in accessibility of these regulatory elements gives rise to differences in phenotype (Buenrostro et al 2015). There are a number of ways in which gene expression can be influenced in *trans*, and one specific casecalled transvection, is a form of regulation dependent on homologous chromosomes being paired (reviewed in Duncan 2002). Throughout this thesis, I have characterized expression patterns at the *Triosephosphate isomerase* (*Tpi*) locus in *Drosophila melanogaster*, establishing *Tpi* as a transvection sensitive locus. This thesis will focus broadly on the creation of *Tpi* alleles that disrupt regulation of the gene resulting in diminished activity in homozygotes, the quantification of mis-regulation due to *trans* effects at *Tpi*, and the demonstration that this up-regulation is pairing dependent. This research complements previous work performed in the Merritt laboratory at the transvection-sensitive locus Malic enzyme, *Men*, which demonstrated a very similar form of transvection, despite having vastly different genomic architecture.

1.2 Gene expression, chromosome structure and genetic regulatory elements

The central dogma of molecular biology holds that DNA is the hereditary material and the morphological diversity seen in the organisms of this world results from differences in genetic sequence, the process of transcription of DNA into RNA, and the translation of RNA into proteins (Crick 1970). While recent advances in the science of molecular biology have expanded heavily on the specifics of transcription and translation and how they are regulated, the central dogma still provides a road map for the generation of a phenotype from DNA.

The process of transcription is manifested in the expression of genes, which have a number of functional and structural characteristics allowing an organism to control gene expression spatially between tissues and temporally through the cell's life cycle. Chromosomal architecture has been widely studied (reviewed in Woodcock 2006); in brief, genes are contained on chromosomes, which are organized into regions which have varying degrees of compaction around histone proteins. Heavily compacted regions of chromosomes are transcriptionally inactive and referred to as heterochromatin. These structures are dense and interactions between regulatory elements and gene promoters occur infrequently. Conversely, euchromatin is less densely packaged which allows binding of transcription factors and expression of genes.

There are a number of regulatory elements which govern how and when genes are expressed; these include promoters, enhancers, repressors and insulators. Promoters and enhancers are regulatory elements that contribute positively to gene expression (reviewed in Stees et al. 2012). Promoter elements are considered start sites for the transcriptional process and are located close to the coding regions with which they are associated and are capable of recruiting basic transcriptional machinery (reviewed in Juven-Gershon and Kadonaga 2010). The deletion of a gene's promoter region is often enough to completely eliminate gene expression at that locus.

While promoters are capable of binding the basal transcription machinery, enhancer elements can significantly increase expression at a locus by encouraging the aggregation and binding of transcription factors around the promoter region, opening chromatin regions around promoters to facilitate access of transcription machinery and/or relocation of chromosomal regions into transcriptionally active regions of the nucleus (Bulger and Groudine 2011). Enhancers are capable of modulating gene expression across large regions of DNA, affecting promoters located on the same chromosome as well as long range interactions on other chromosomes (Williams et al 2010).

While promoters and enhancers participate in positive regulation of gene expression, the vast number of genes in an organisms' genome require factors that can negatively influence gene expression. These factors can be broadly categorized as either repressors or insulators. A repressor is a protein which is capable of binding DNA in such a way as to reduce accessibility to that genomic region, or to prevent the assembly of transcriptional machinery at that point (reviewed in Hanna-Rose and Hansen 1996, Payankaulam et al. 2010). One of the larger eukaryotic families of repressor proteins is the Polycomb group proteins. Polycomb group repressors act by inducing methylation of histone H3 or ubiquination of histone 2A (Morey and Helin 2010), both of these processes being markers for inactivation of chromosomal regions resulting in repression of expression of genes in the affected region. Repressor proteins are dynamic, moving throughout the genome and binding specific loci in a time dependent manner, as opposed to insulators which are genetic sequences whose genomic location is static. A recent bioinformatics-based study of insulator elements in *Drosophila* (Negre et al. 2010) classed insulators into two fairly distinct categories; class I and class II insulators. Typically, class I insulators are binding sites for BEAF-32, CP190, and CTCF, are located at the boundaries

between genes with distinct transcriptional profile differences, and often represent boundaries between heterochromatic and euchromatic regions. Class II insulators tend to be bound by Su(Hw) proteins, however, the functional significance of class II insulators is still relatively unknown. Despite recent advances in our understanding of class I insulators, the function of insulators in general is not heavily agreed upon (Schwartz et al. 2012), with recent evidence suggesting even the enhancer blocking activity of insulators may be context-dependent. In addition, while relatively simple transcription elements regulate interactions at specific loci, large scale organization of chromosomes can influence transcription in a number of complex ways.

1.3 Nuclear Organization

The genetic material of an organism is vast in comparison to the nuclear compartment it occupies. This size disparity requires rigorous yet dynamic control over how chromosomes are packaged. Chromosomes must be packaged in such a way that the genetic material all fits, yet can also be accessed when expression is required. Through the use of modern visualization techniques (3C, 4C and FISH), researchers have been able to visualize all of the chromosomes in an organism simultaneously, which showed a clear structured organization of genetic material into specific regions of the nucleus (Bolzer et al 2005). As the visualization techniques continued to improve, it became clear that spatial organization is dynamic and that chromosomes move within their regions allowing a number of complex interactions (reviewed in Gondor and Ohlsson 2009, Cremer et al. 2015). This organization allows chromosomes to interact with themselves (in *cis*) as well as interact with each other (in *trans*) at boundaries between chromosome regions. Although chromosomes are able to interact in *cis* as well as in *trans*, the organization of most eukaryotic nuclei places homologous chromosomes in different regions,

limiting *trans* interactions. In dipteran insects, however, this is not the case as interphase homologous chromosomes are paired (Mckee 2004). A growing body of evidence suggests a diversity of organisms use chromosomal interactions to shape their spatial architecture and transcriptional profile including yeast (Mirkin et al. 2013), mice (Lomvardas et al. 2006, Zhao et al. 2006), as well as the model organism of this study, *Drosophila* (Reviewed in Duncan 2002). Among the many forms of chromosomal interactions, there is one type that relies on the pairing of homologous chromosomes in somatic tissues. This particular type of communication between paired chromosomes in *trans* is called transvection.

1.4 Gene regulation in cis and trans, transvection.

The expression of a gene is a complex process involving enhancers, repressors, insulators and RNA polymerases accompanied by their associated transcription factors. These interactions are further mediated by the arrangement of chromosomes in the nucleus. The dynamic movement of chromosomes allows for interactions between promoters and the supporting elements in both *cis* and *trans* (Gondor and Ohlsson 2009). The structural organization of chromosomes largely influences how they are regulated, not only in terms of the location of genetic elements along the chromosome, but also their spatial location within the nucleus. This spatial organization is exemplified by the apparent expression gradient as gene-rich regions of chromosomes are positioned further toward the interior of the nucleus, although some transcriptionally active portions of chromosomes are moved temporarily toward nuclear pores (reviewed in Cremer et al. 2015). Cis interactions that control gene expression have been well characterized and the *β-globin* locus in mammalian cells is a well-studied example. Briefly, the *β-globin* locus is controlled by a combination of enhancer action in *cis* through the binding of transcription factors to the Locus Control Region (LCR) and looping action which loops out the heterochromatic

region between the LCR and the currently active promoter, bringing the transcription machinery as close as possible to the specific promoter (reviewed in Stamatoyannopoulos 2005). There are a number of different promoters the LCR is able to activate, each one arranged in sequence without its own enhancer region. The LCR will interact with a different promoter depending on the developmental stage of the cell. The classical concept of regulation of gene expression in *cis* being the sole driver of gene expression has, however, come into question as evidence for interactions between chromosomes grows in a number of organisms (reviewed in Williams et al. 2010). These interchromosomal interactions include control of gene expression (Spilianakis et al 2005), imprinting of epigenetic states (Sandhu et al. 2009) and potentially inactivation of X chromosomes (Xu et al. 2006, 2007). The interactions between chromosomes in *trans* are more difficult to elucidate than those in *cis* because chromosomes tend to prefer to interact in *cis*, often requiring special situations to interact in *trans*. This tendency to act in *cis* is termed *cis*-preference (Geyer et al. 1990).

In *Drosophila*, the paired nature of homologous chromosomes in somatic cells allows for pairing-dependent interactions in *trans*, or transvection. Transvection, which can drive up-, or down-, regulation of a certain locus (reviewed in Duncan 2002). Despite the typical preference of enhancers to act on promoters in *cis*, there is growing evidence that some pairing relationships show competition for enhancer elements between promoters in *cis* and *trans* (Morris et al 1999, Bateman et al. 2012). Mechanistically speaking there are two main ways transvection can affect regulation of a gene through the pairing of homologous chromosomes: the bypass of chromatin insulators in *cis*, and the long distance enhancer action in *trans* (reviewed in Duncan 2002, Figure 1).

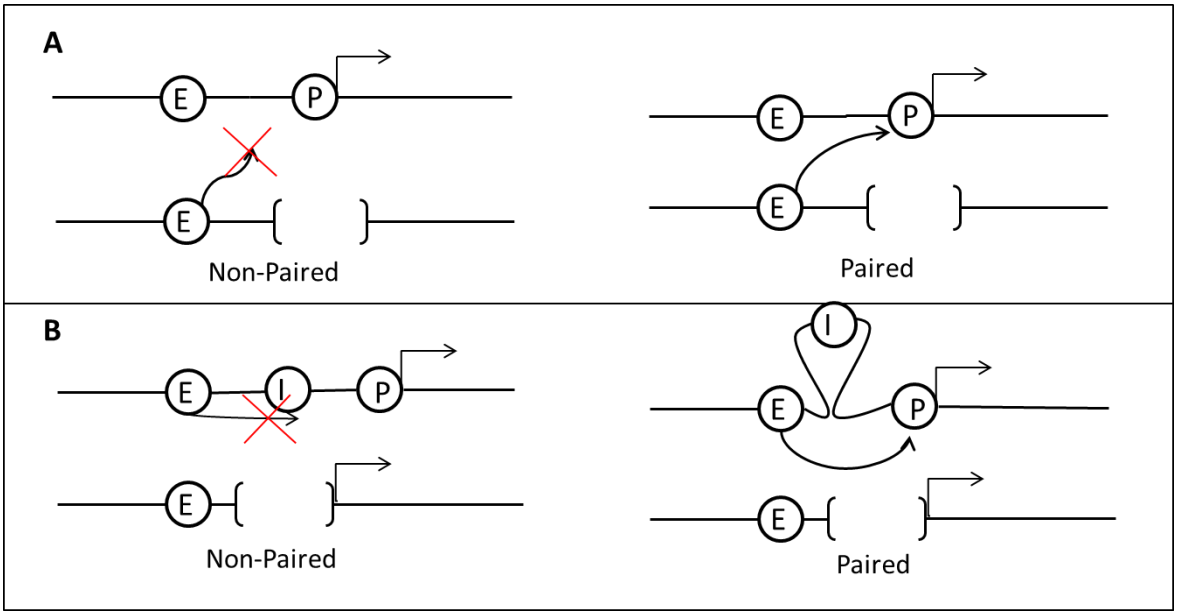

Figure 1 (A) Schematic diagram of the long distance enhancer action in trans model of transvection. When homologous chromosomes are paired enhancer elements are able to act on the functional homolog. (B) Schematic diagram of the bypass of chromatin insulators in cis model of transvection. A genetic lesion removes a large piece of the regulatory region, pairing of the homologs then loops out the insulating region, which is no longer homologous. Figure was adapted from Morris et al (1998).

Pairing dependent *trans*-interactions were first described at the Ultrabithorax complex (BX-C) (Lewis 1954). In this landmark paper, it was noted that two different mutant alleles bx^{34e} and Ubx^{1}, which produce a mutant phenotype when homozygous, would complement each other when heterozygous to form a wild-type phenotype, while flies homozygous for either mutation show a distinct mutant phenotype. After discovering transvection at BX-C, Lewis continued to characterize the interactions between chromosomes in this region. Lewis went on to find that rearrangements disrupted the phenotypic rescue in bx^{34e}/Ubx^{1}; these rearrangements were able to disrupt pairing (confirmed by polytene chromosome analysis), proving the pairing dependent nature of this complementation. Lewis then went one step further, showing that the rearrangement could be on either chromosome and still disrupt phenotypic rescue, but the

rearrangement had to fall within the region between the centromere and the BX-C complex. Finally Lewis showed that a double rearrangement (R(bx^{34e})/R(Ubx^{1})) re-established pairing at the BX-C complex, allowing for phenotype rescue through transvection. Further research into the genes that make up the ultrabithorax complex have shown that the region is very permissive to transvection, but is also extremely rich in regulatory elements which drive a variety of complex forms of complementation. (Castelli-Gair et al. 1990, Goldsborough and Kornberg 1996, reviewed in Duncan 2002).

Another example of enhancer action in *trans* driving an up-regulation of a homologous promoter has been well characterized at the *yellow* locus in *Drosophila*, a gene involved in the pigmentation of various adult structures. Transvection at the *yellow* locus was first characterized by Geyer et al. (1990) who showed that two alleles which did not produce functional pigment as homozygotes were able to produce functional pigmentation when heterozygous. After testing several different combinations of *yellow* knockouts, complementation at the *yellow* locus was found to be possible in one of two ways, either enhancer action in *trans* where wing and body enhancers act on the homologous promoter which does not have a functional enhancer region, or through bypass of insulator in *cis* where the pairing of the homologous allele loops out an insulator which had been blocking enhancer action in *cis*, allowing restoration of transcription (Morris et al. 1998, 1999, Figure 1).

While examples of transvection driving up-regulation of transcription are quite common, there are also situations where transcription at a locus is repressed in a pairing dependent way. A well characterized example comes from Chen and Pirrotta (1993) in which the X-chromosome linked eye-color gene *white* is repressed by aggregation of proteins at the *zeste* locus. This suppression of *white* is driven by a mutation in *zeste* (z^{1}) first described by Gans (1953) that causes hyper-

aggregation of proteins at *zeste* binding sites in the eye-specific enhancer region. While the mutant allele z^1 causes a decrease in *white* expression in females, z^1 males show normal *white* expression. This difference is driven by the presence of the Y chromosome in males, which does not have the eye enhancers for z^1 to aggregate on, and only a single X chromosome. The reduction in accumulated protein is enough to allow the transcriptional machinery to bind to the *white* locus (Chen and Pirrotta 1993). Supporting the hypothesis that hyper-aggregation is causing the repression of *white* expression, Chen created another mutant z^{op6} which aggregates even more readily than z^1. Further research showed that the z^{op6} allele is capable of repression of *white* expression in both males and females (Lifschytz and Green 1984). Since the *white* gene is located on the X-chromosome it also represents a sex-specific form of transvection: only females will have a homologous X chromosome with which to pair. While the paired nature of homologous chromosomes in *Drosophila* lends itself heavily to *trans*-interactions, in order for these interactions to be considered transvection they must depend on pairing to function. As such, the elimination of *trans* effects when pairing is disrupted is the currently accepted assay used to determine if the differences in expression seen at a locus are, in fact, transvection.

1.5 Pairing of homologous chromosomes and transvection

In order for an interaction between chromosomes to be called transvection, the effect of the interaction must be diminished when the pairing between homologs is disrupted. When transvection was first discovered at *Ubx,* Lewis was able to disrupt transvection by introducing rearrangements between the centromere and the Ultrabithorax complex, in which *Ubx* resides. The disruption of pairing was confirmed by observing polytene chromosomes (Lewis 1954) and this established that a "critical region" had to be intact for pairing. The proximal limit of this critical region seemed to consistently be at the centromere, leading to the hypothesis that pairing

initiates at the centromere, and it would follow that a rearrangement between the centromere and a gene would be sufficient to disrupt pairing at that locus (Lewis 1954). As visualization techniques advanced, this hypothesis fell out of favour as independent studies on *Drosophila melanogaster* chromosome arm 2L (Fung et al. 1998) and chromosome arm 3R (Gemkow et al. 1998) showed that pairing initiates at multiple sites along the chromosome arm and maintains an equilibrium with the dissociated form.

Further support for a more complex model of homologous pairing arose when other transvection-sensitive alleles were examined. Examination into the interactions between homologous chromosomes at the *white* locus showed a much more restricted relationship (Gans 1953, Smolik-Utlaut and Gebart 1987), as no rearrangements tested could disrupt repression of *white* caused by the mutant z^1 allele. Another study on the nature of pairing at the *white* locus used duplications to increase the distance between the *zeste* binding sites and the *white* allele on one chromosome but not the other, functionally disrupting the homologous association rather than disrupting pairing (Jack and Judd 1979). These authors found that moving one copy of the *white* gene further away was sufficient to disrupt repression. Thus, repression of *white* in *zeste* mutants is dependent on both homologous loci being in close enough proximity for the aggregation of zeste proteins to block transcriptional machinery. As more detailed investigations of transvection sensitive loci are performed, a more complex and complete picture of the basic workings of transvection is developing. The transvection sensitive locus at *Tpi* represents a promising new case to investigate, due to several unique features in its genomic architecture.

1.6 Transvection at the *malic enzyme* locus, *Men*

Higher than expected malic enzyme (MEN) activity in *D. melanogaster* heterozygous for a knockout and wild type allele suggested that this locus was susceptible to interchromosomal

interactions and regulation (Merritt et al 2005). In order to investigate these *trans* effects more thoroughly a series of alleles with small lesions at the *Men* transcriptional start site were created using *P*-element deletion mutagenesis (Lum and Merritt 2011). These alleles excised varying amounts of genetic material and were shown to have significant differences in their ability to up-regulate *Men* in *trans* (Lum and Merritt 2011). This up-regulation was then proven to be pairing dependent (Bing et al 2014), establishing *Men* as a transvection-sensitive locus. The amount of up-regulation at *Men* was also shown to be significantly affected by genetic background (Lum and Merritt 2011) and environmental change (Bing et al. 2014), where a single excision allele would show varying levels of transvection effects in *trans* with different backgrounds or following changes to temperature. Transvection at *Men* is believed to be driven by long distance enhancer action in *trans* in which regulatory elements from the knockout allele drive up-regulation of an intact homolog (Lum and Merritt 2011). While the *Men* locus is transvection sensitive, the large coding and regulatory regions make studying subtle effects difficult, so a smaller gene with a more restricted regulatory region is preferred such as the *Tpi* locus.

1.7 The *Triosephosphate isomerase* locus, *Tpi*

The *Tpi* gene, located on chromosome arm 3R, is only approximately 1.5kb in length making it the shortest gene identified to date showing transvection (excluding *Salivary glue secretion-4* which is only expressed briefly in the larval salivary glands; Muskavitch and Hogness 1980). *Tpi* encodes the metabolic protein triosephosphate isomerase (TPI) which catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) into glyceraldehyde-3-phosphate (G3P). The *Tpi* gene represents a particularly attractive target for transvection studies since, in addition to being a very short gene, it also has an extremely short regulatory region (Figure 2). This

regulatory region falls just downstream of a predicted class I insulator, which should block any enhancer action in *cis* from upstream regulatory elements. Despite extensive research in other organisms there have been very few mutant *Tpi* alleles generated and characterized in *Drosophila melanogaster*. The objective of this thesis was to develop *Tpi* as a novel model of transvection to complement work already done at *Men* and other loci.

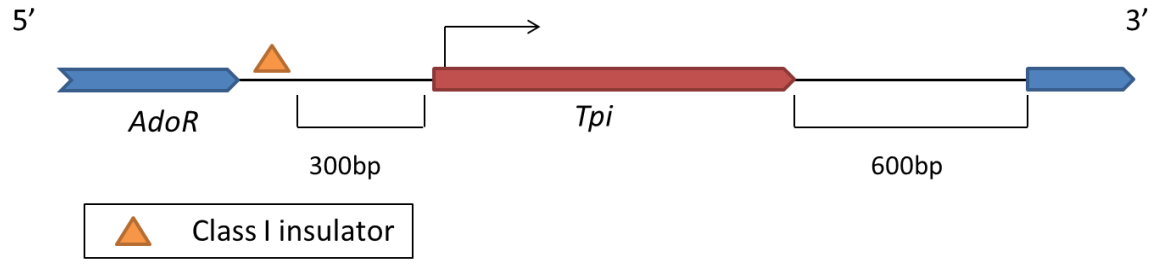

Figure 2 Schematic diagram of the *Tpi* gene region and the surrounding genomic architecture. The *Tpi* gene lies in a very gene rich area and as such has a very small regulatory region.

Chapter 2 Characterization of the transvection sensitive locus *triosephosphate isomerase* in *Drosophila melanogaster*.

2.1 Introduction

Expanding our understanding of the regulation of gene expression is an important part of the development of a functional relationship between genetic sequence and phenotype. These advances in understanding have allowed for the discovery of a number of complex phenomena which regulate the expression of genes through a number of interacting elements. The elements, which interact with each other and the genome to regulate gene expression, can be grouped broadly into four groups; promoters, enhancers and their associated transcription factors, repressors and insulators. Promoters and enhancers are elements which have a positive effect on gene expression, while repressors and insulators work to repress and sequester genes respectively.

For many years it was believed that regulatory elements were only capable of interacting in *cis* (on the same chromosome), but interactions in *trans* have been found to be common throughout a growing list of organisms including yeast (Mirkin et al. 2013) and mice (Lomvardas et al. 2006, Zhao et al. 2006), as well as the model organism of this study, *Drosophila* (Reviewed in Duncan 2002). Despite the presence of *trans* interactions in a number of organisms the compartmentalization of homologous chromosomes in most eukaryotic somatic cells (Bolzer et al 2005) makes the number of possible *trans* interactions very small when compared to the overwhelming number of *cis* interactions. Compartmentalization of homologous chromosomes in the soma is not, however, universal and somatic homologs are, in fact, paired in *Dipteran* species (Mckee 2004). This pairing of homologs allows for a special form of interaction in *trans* called transvection, which is a pairing-dependent mis-regulation of gene expression. While transvection

appears to be possible at any location in the *D. melanogaster* genome (Mellert and Truman 2012), the preference for interactions in *cis* (termed *cis*-preference, (Geyer et al. 1990, Bateman 2012)) often limits transvection to special situations. These special situations and the resulting transvection effects have been studied at a series of loci including the ultrabithorax complex (Lewis 1954), *yellow* locus (Geyer et al. 1990, Morris et al. 1998,1999) and the *white* locus (Gans 1953, Smolik-Utlaut and Gebart 1987) and *malic enzyme* (*Men*) (Merritt et al. 2005, Lum and Merritt 2011).

In this study we characterized transvection at the *triosephosphate isomerase* (*Tpi*) locus, which is one of the smallest transvection sensitive genes discovered to date. *Tpi* encodes the metabolic protein triosephosphate isomerase (TPI) which catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) into glyceraldehyde-3-phosphate (G3P). The *Tpi* gene represents a particularly attractive target for transvection studies since besides being a very short gene it also has an extremely short regulatory region at 300bp. We find that transvection at *Tpi* behaves very similarly to transvection at *Men* and presents evidence for the presence of a possible critical region for pairing through the use of rearrangement chromosomes. My results suggest a model of transvection in which enhancer elements are shared in *trans* as opposed to a looping out of insulating factors in *cis*. Transvection at the *Tpi* locus is also found to vary significantly with differences in genetic background, which makes up the rest of an organism's genome beyond the locus of interest. Further analysis indicates that transvection at *Tpi* is dependent on a critical region for pairing which extends from the centromere of chromosome 3 into the middle of chromosome arm 3R, consistent with that of *Men* and the ultrabithorax complex. Continued research into how transvection occurs at different loci will help shape our

understanding of interactions between chromosomes in *trans* and allow for more thorough models of gene regulation based on the genetic sequence.

2.2 Materials and methods

Fly stocks

Isothird chromosome lines for genetic background studies, VT26, CT21 and HFL32 and VT83, are a subset of non-lethal third chromosome lines extracted previously (Duvernell and Eanes 2000; Merritt et al. 2005). The other genetic background line (#6326) was obtained from the Bloomington Drosophila stock center (BDSC). BDSC stock line 16563 (insertion EY03361) was used in *P*-element excision- based mutagenesis experiments to create small lesions initiating at an insertion site at the *Tpi* gene transcriptional start site to disrupt expression. The transposase for *P*-element mobilization was provided by crossing selected fly lines to the BDSC stock line 2030. *P*-element mutagenesis was performed following the protocol of Lum and Merritt (2011). Inversion chromosome-containing lines were obtained from the BDSC (stock #106300, 30913).

Collection of Flies for experiments

Flies for enzymatic and transcription assays were generated by mating 5-10 day old adult flies with a total of 5 female and 3 male flies in each vial. Each cross was replicated across 4-6 vials with adult flies being cleared 5 days after mating. Male flies were collected during the day they eclosed and reared on cornmeal medium at 25°C with a 12:12 light cycle for 4 days prior to being assayed or frozen down by the end of the fifth day. Frozen flies were kept at -80°C for a maximum of four weeks prior to being assayed. Samples of 5 male flies were used for all experiments.

TPI enzymatic activity

Enzyme activity assays consisted of a minimum of 5 samples and a maximum of 8 from each genotype isolated from between 4-6 vials. Fly samples were homogenized in 500µL homogenization buffer using physical disruption followed by centrifugation and the supernatant was collected for analysis. The homogenization buffer consisted of a solution with 0.1M Tris-HCl pH 7.4 and 0.15mM NADH. TPI enzymatic activity was measured using 10µL of supernatant in 100µL of assay solution. The assay solution consisted of 0.1M Tris-HCl, 0.15mM NADH, 0.83mM glyceraldehyde-3-phosphate, and 2 units per mL of glycerol-3-phosphate. Absorbance at 320nm was measured at 9 second intervals over the course of 3 minutes with an optical density cut off of 0.05 and the activity was obtained by the slope of the absorbance plot. Each sample was assayed in triplicate and mean activity was used for statistical analysis. Total soluble protein content was measured using the Applied Biosystems Pierce BCA Protein Assay kit according to the manufacturer's instructions (CAT#23225) and used to control for homogenization differences. ANOVA was performed on total soluble protein controls to ensure no variation in protein load. Analysis of enzymatic data was then carried out using ANOVA to detect significant differences in mean, followed by Tukey's honestly significant difference (HSD) test to determine specific significant differences between samples and expected values. Expected values were calculated using in plate controls to determine homozygous activity then the formula [$(\text{TPI activity}_{\text{allele1}} + \text{TPI activity}_{\text{allele2}})/2 = \text{Expected heterozygote activity}$] was applied.

Nucleic acid isolation

DNA and RNA were isolated from flies of each genotype in triplicate, with each sample being taken from a separate replicate vial. RNA for real time quantitative polymerase chain reaction (RT-qPCR) was isolated using the Qiagen RNEasy Plus RNA isolation kit (Qiagen CAT #74134), cDNA synthesis was performed on each independent sample using Applied Biosystems' High Capacity cDNA synthesis kit (Life Technologies CAT # 4368814), and the quantity and purity of the RNA/cDNA were determined using Thermo Scientific ND8000 Nanodrop. DNA for sequencing was isolated using the Qiagen DNeasy Blood and Tissue Kit (CAT # 69506) and amplified using Qiagen TopTaq Master Mix kit. The DNA with the highest quality was used for sequencing reactions performed by Genome Quebec's Nanuq Sequencing service.

RT-qPCR

RNA samples for use in multiplex qPCR were taken from three independent vials of the same genotype, these three RNA samples were then reverse-transcribed into cDNA and used as three biological replicates. RT-qPCR was then performed on each cDNA sample in quadruplicate technical replication, as well as a no-RT control to ensure amplification was not due to genomic DNA (gDNA) contamination. Each reaction (total volume of 20µL) was composed of 0.4µl cDNA, 7.6µL water and 1µL of each premixed primer-probe combination and 10µL Taqman Gene Expression Master-mix (Life Technologies CAT # 4370048) using the Eppendorf Mastercycler ep realplex 4 thermocycler. Reaction conditions were 2 minutes hold at 50°C, 10 minutes hold at 95°C and 40 cycles of 10 seconds at 95°C followed by 1 minute at 60°C. Transcription at *Tpi* was measured using a custom primer probe set purchased from Integrated DNA technologies (Primer 1: GGA CCT CTT GAG CCT GAT, Primer 2: CCT ACG CCC AGA

AGA TCA A, Probe: /56-FAM/TAG GCC ACC /ZEN/ACC ACG TTC TTC C/3IABkFQ/), and was quantified relative to *rpl32* expression measured using Life Technologies Taqman Gene Expression kit (Life Technologies CAT #4448489). Amplification efficiencies of the RT-PCR primers were validated using the procedures outlined in Livak and Schmittgen (2001). The analysis of qPCR results was performed in Microsoft Excel using the formulae for the ΔΔCt method detailed in the Applied Biosystems "Guide to Performing Relative Quantification of Gene Expression Using Real-Time Quantitative PCR" (Applied Biosystems 2008) and quantified relative to the wild type allele *TpiExF*.

2.3 Results

P-element excision-derived *Tpi* alleles

We generated a suite of excision-based *Tpi* alleles that varied in the size and location of the genomic excision and the amount of *Tpi* expression and TPI activity. *P*-element-mediated excision of inserted element P{EPgy2} in the BDSC stock line 16563 resulted in four low activity *Tpi* alleles (*TpiExL1*, *TpiExL2*, *TpiExL3*, and *TpiExL4*). One medium activity allele (*TpiExM*) was also generated for this experiment along with one wild-type expression allele (*TpiExF*). Homozygous activity of all excision alleles is represented in Figure 3. Letters indicate significant differences between the groups (Tukey's HSD, $\alpha=0.01$, $k=7$, $df=49$). DNA regions flanking the insertion site were amplified and sequenced to establish excision site boundaries. Sequence data indicates *TpiExF* is a perfect excision of the *P*-element with no damage to the surrounding genetic material. The remaining *Tpi* excisions (*TpiExM,L1,L2,L3 and L4*) excise some portion of genetic material between 500bp upstream and 650bp downstream of the transcriptional start site, although the precise nucleotides within that range which have been affected are unknown.

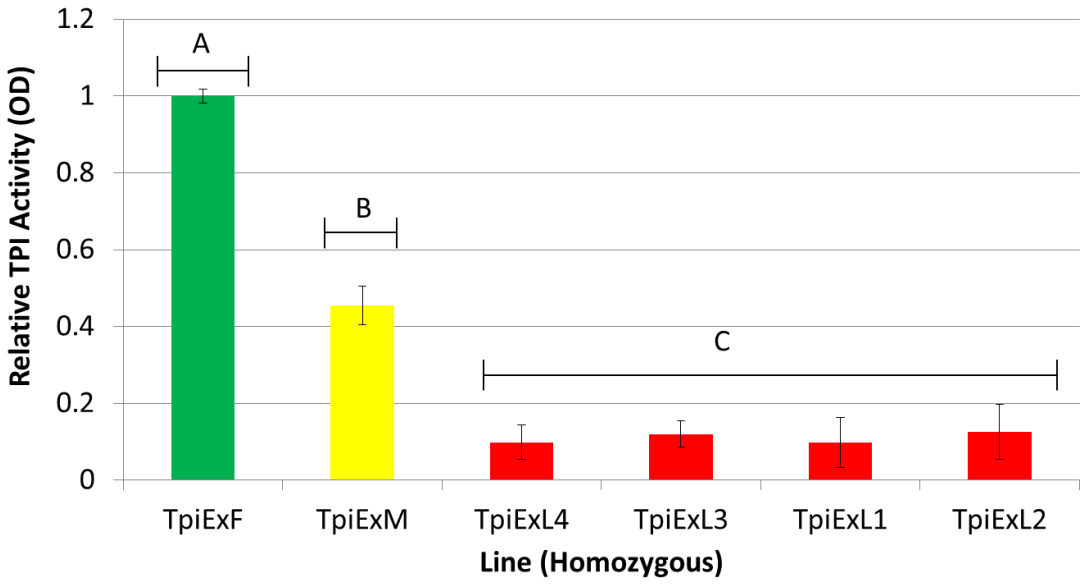

Figure 3 Homozygous TPI activity for the *P*-element excision lines shown relative to *TpiExF*. Bars in red were below the limit of detection for the TPI activity assay and have been labeled "low activity" alleles. Letters represent significance groups at α=0.01 (Tukey's HSD k=7, df=49).

The TPI kinetic enzyme assay is an accurate indicator of TPI activity and *Tpi* expression.

In order to efficiently screen and assay a large number of fly samples, a kinetic absorbance assay of protein activity is preferred (simpler and less time-intensive than assays of transcription). In order to confirm that the differences we observed in TPI activity reflect *Tpi* expression we had to demonstrate that our kinetic assay accurately reports enzyme activity. If TPI enzyme activity is indeed an accurate predictor of expression at the *Tpi* locus, then we could accept its use to study transcriptional phenomena.

In order to determine if the TPI activity assay can differentiate between subtle differences in activity, a dilution standard curve was performed (Figure 4). The results of the dilution series indicated that differences in enzyme activity as low as 10% can be reliably resolved by our assay (R^2=0.8440). When we compared TPI activity with mRNA expression in the same sample, we

found that the two were positively correlated (R=0.7924) (Figure 5). Each point on the graph represents the kinetic enzyme activity and expression at *Tpi* for homozygous alleles. Values have been made relative to the *TpiExF* wild-type line. As predicted by the kinetic enzyme assay, homozygous low activity alleles also show very low levels of expression of *Tpi* relative to the wild type allele, essentially at the lowest level detectable, but repeatedly with some level of expression. For this reason, we class these alleles "low" activity and not knock-out, although the level of residual gene expression is almost negligible.

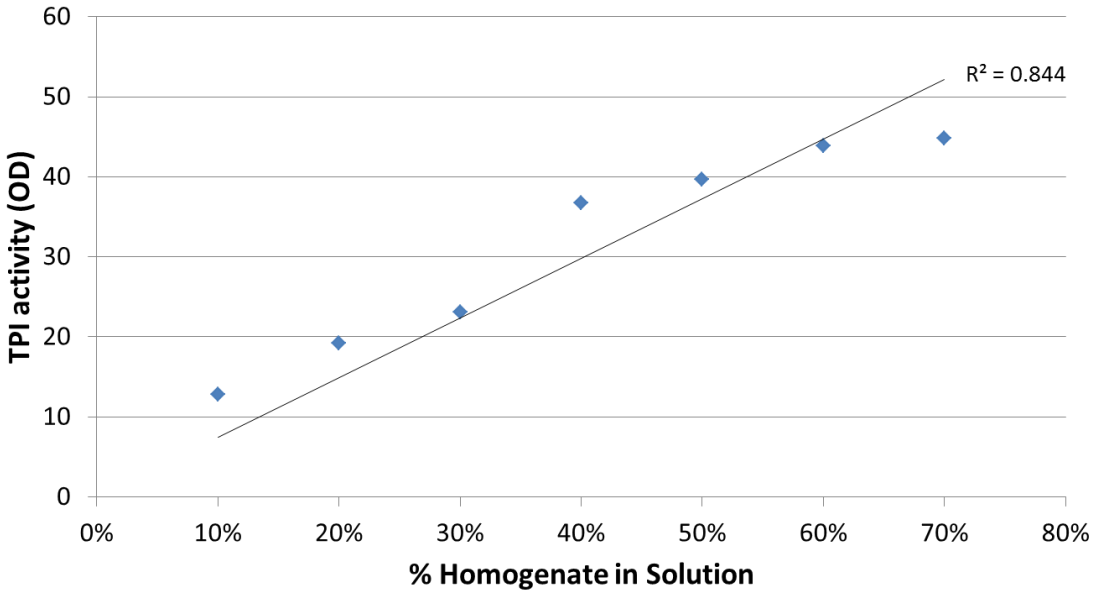

Figure 4 TPI activity values for a dilution series using whole fly homogenate from homozygous *TpiExF* flies diluted in homogenization buffer. Predicated line has a y-intercept of 0 OD units R^2=0.844.

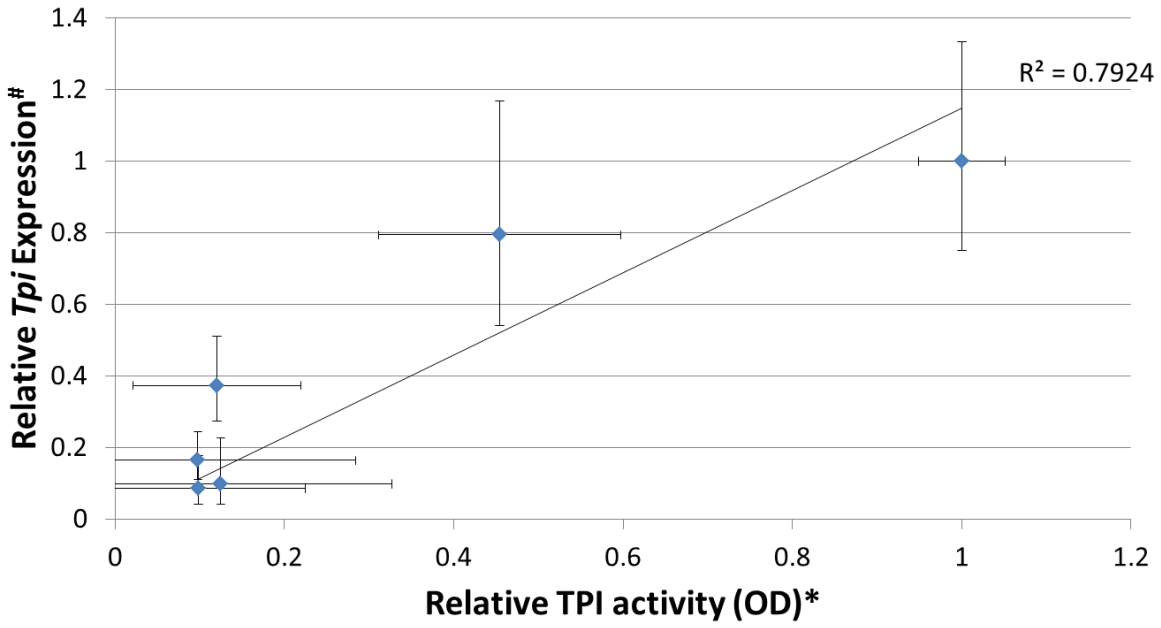

Figure 5 Graph representing homozygous TPI activity on the x-axis with error bars for Relative Standard Deviation and homozygous *Tpi* expression on the y-axis with error bars for activity range, R^2=0.7924. Low activity alleles pool together at the lower thresholds of both expression and activity assays.

<u>Trans-interactions at the *Tpi* locus are present and dependent on genetic background.</u>

Trans-interactions between paired homologous chromosomes are known to drive increased expression at loci in the *Drosophila* genome (reviewed in Duncan 2002), and it follows that a locus sensitive to *trans*-interactions will usually demonstrate non-additive expression levels. Homozygous activity for each allele was used in the formula [(TPI activity$_{allele1}$ + TPI activity$_{allele2}$)/2 = Expected heterozygote activity] to determine the expected additive expression value. Trans-interactions are, however, generally superseded by cis-interactions if the promoter region is intact, and cis-interactions are expected to result in additive activity at that locus. To determine if the *Tpi* locus has non-additive expression indicative of *trans* interactions we crossed female *TpiExF* flies to males of each other excision allele and assayed the heterozygotes for TPI

activity. Our results demonstrate higher than additive activities, i.e. *trans*-interaction between low activity alleles and the functional wild-type promoter (Figure 6). The activity of alleles *TpiExL1*, *2*, *3* and *4* are significantly greater than the expected value (50% of wild-type), while putative cis-only regulatory alleles *TpiExF* and *TpiExM* show expected values (wild type and 75% wild-type respectively). Our results also suggest that the amount of up-regulation, the degree of transvection, varies between *Tpi* excision alleles. This is consistent with our examination of transvection at the *Men* locus, which shows a strong dependence on the amount of transvection on the sequence of the excision allele, although the differences between excision alleles at *Tpi* are not always significant and are much less pronounced.

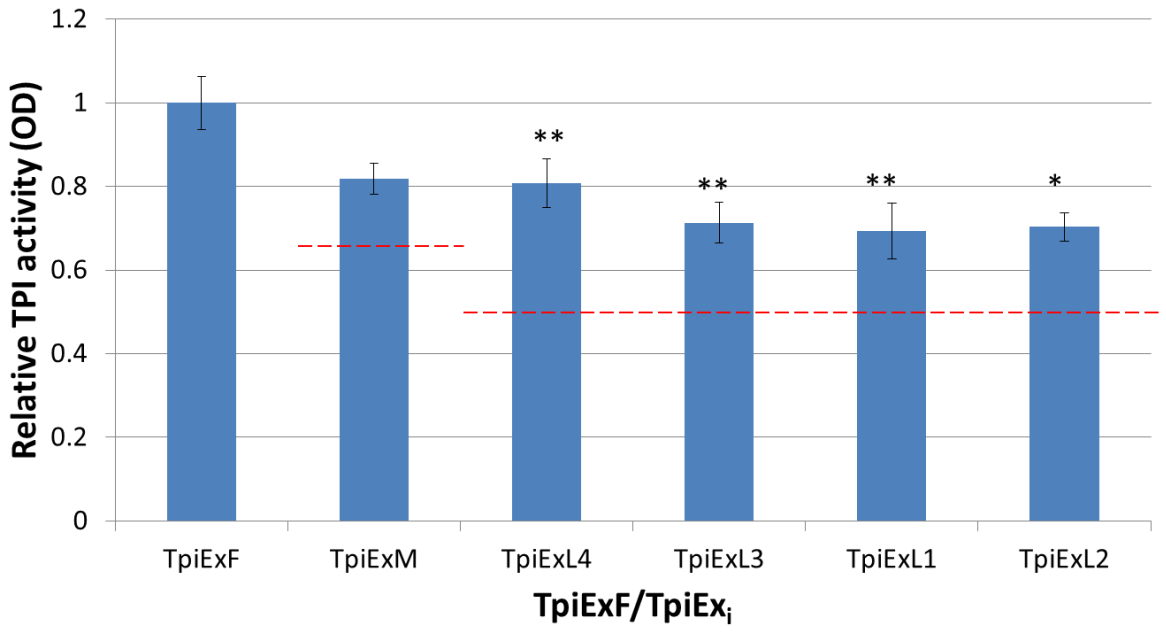

Figure 6 Relative TPI activity for individuals heterozygous for a wild type (*TpiExF*) allele and an allele with a damaged promoter. * Indicates significant difference from expected value at α=0.05,** at α=0.01, Tukey's HSD k=7, df=40. The dashed red line represents expected additive TPI activity.

It has previously been shown that the amount of up-regulation, i.e. the degree of transvection, is sensitive to both the excision allele and genetic background (Lum and Merritt 2011, Bing et al. 2014). In order to determine if trans-interactions at *Tpi* also vary based on genetic background, each knockout allele, the mid-activity allele *TpiExM* and the wild-type *TpiExF* were crossed to three genetic backgrounds (6326/VT83;TpiEx$_i$/VT26, 6326/VT83;TpiEx$_i$/CT21 and 6326/VT83;TpiEx$_i$/MD76). Results indicate up-regulation due to trans-activity varies significantly based on genetic background (Figure 7). Interestingly, the *TpiExM* allele shows significant differences in TPI activity despite not being a complete knockout.

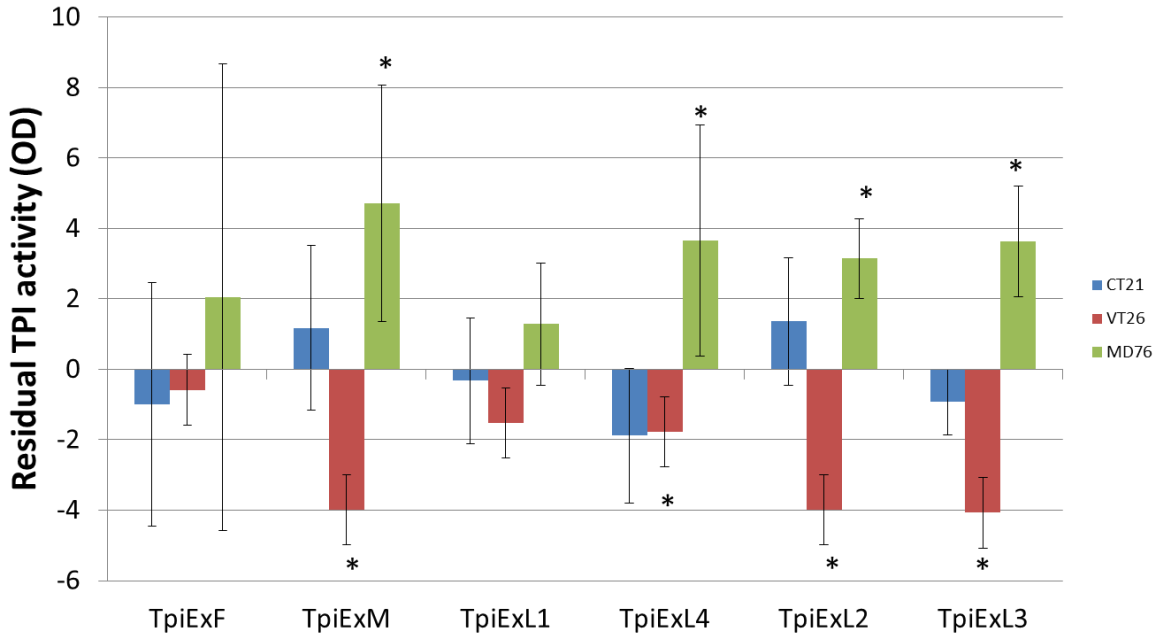

Figure 7 Graph showing residual TPI activity after subtracting the average activity of each allele pooled across all three backgrounds. Error bars represent standard deviation. * indicates significant difference from mean across all three backgrounds, two-tailed t-test assuming unequal variances α=0.05.

Trans interactions at *Tpi* are transvection.

In order to determine if the *trans* effects we observe at *Tpi* are indeed transvection, we had to determine if the up-regulation in these heterozygotes is pairing dependent. The standard method of determining if trans-effects are pairing dependent is to use inversion chromosomes or rearrangements to disrupt pairing in heterozygotes (Duncan 2002; Bing et al 2014). To this end, we created heterozygotes with our *Tpi* excision alleles and two different inversion chromosomes In(3LR)LD6 and In(3LR)Tl^{rv18} by crossing our excision allele lines to BDSC stocks lines 106300 and 30913, respectively. We used two different inversions because previous work has shown that the relationship between loss of transvection effects and the location of inversion breakpoints is not necessarily straightforward (Bing et al 2014). Chromosome In(3LR)LD6 is a pericentric inversion with an upstream breakpoint located within 62A10-62B1 and a downstream breakpoint between 85A2-85A3 (Figure 8A) and has been shown to disrupt transvection at *Men* (Bing et al. 2014). The second Inversion chromosome used (In(3LR)Tl^{rv18}) has an upstream breakpoint at 74A and a downstream breakpoint between 97D1-97D2 (Figure 9A). Flies heterozygous for inversion In(3LR)LD6 and an excision allele showed expected values for an allele regulated in *cis* indicating elimination of *trans* activity driving up-regulation of *Tpi* expression (Figure 8B). These results are consistent with previous work at *Men* (Bing et al. 2014). Interestingly, flies heterozygous for inversion chromosome In(3LR)Tl^{rv18} and an excision allele results in varying levels of pairing disruption (Figure 9B) as several alleles reported higher than expected TPI activity. As stated, different inversions have been shown to result in different levels of disruption of transvection, presumably through differences in the degree to which they disrupt pairing. Given this explanation, the less pronounced, and more variable, effect of In(3LR)Tl^{rv18} (the inversion with the downstream breakpoint of much closer proximity to the *Tpi* gene) is surprising and speaks to the complex relationship between chromosome architecture,

pairing, and trans-interactions. Additionally, the allele *TpiExM* has a significantly greater than expected TPI activity despite being a seemingly *cis* only regulated allele, indicating possible *trans* activity between the inversion chromosome and this excision allele.

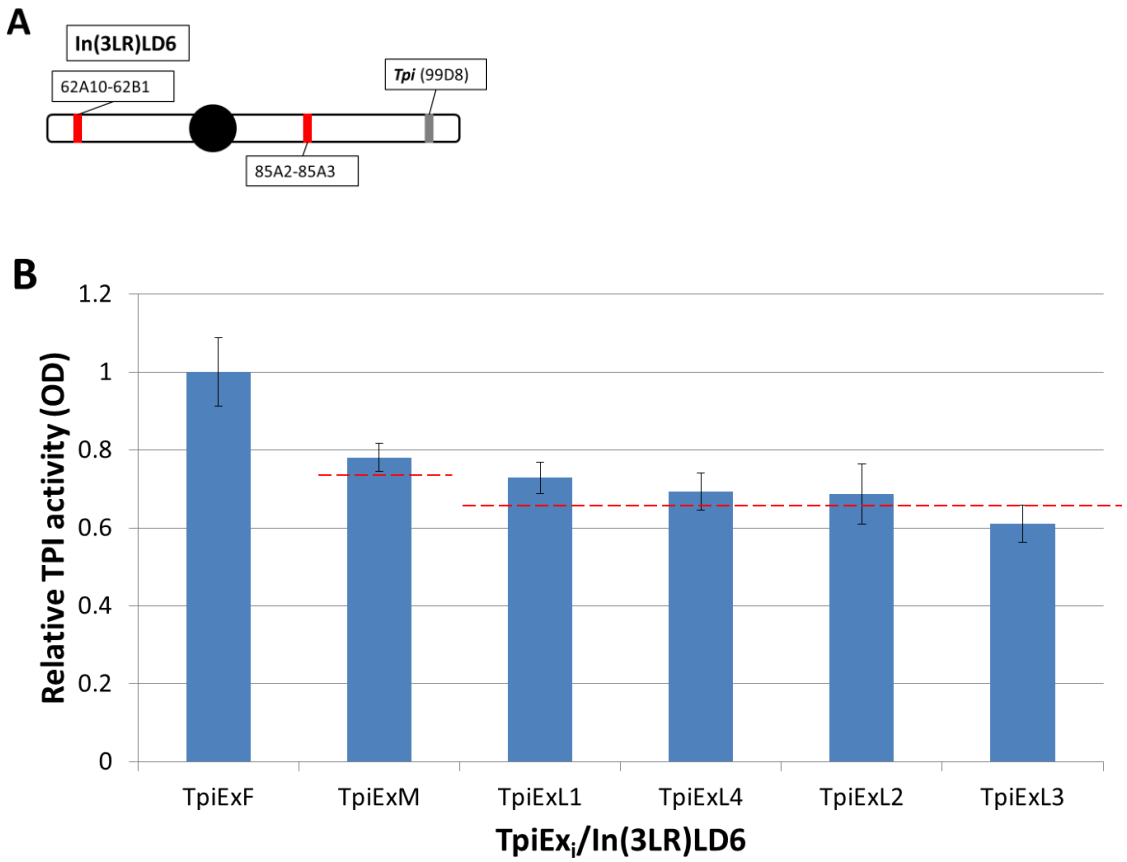

Figure 8 (A) Schematic diagram showing the breakpoints of inversion chromosome In(3LR)LD6 and the location of the *Tpi* locus. (B) TPI activity levels for flies heterozygous for an excision allele and the inversion In(3LR)LD6. All lines show activity levels expected for enhancer action in *cis*-only suggesting the up-regulation seen previously is transvection. Dashed red line indicates expected activity for all In(3LR)LD6/Low activity heterozygotes.

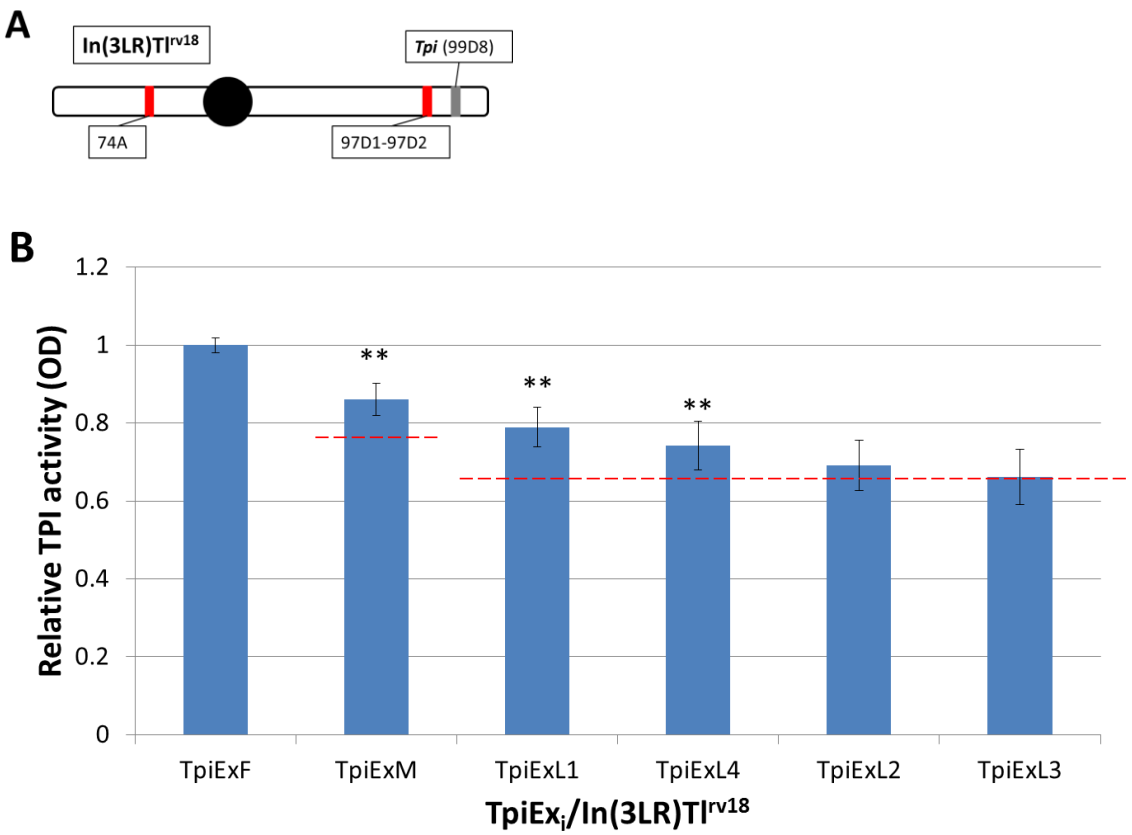

Figure 9 (A) Schematic diagram showing the breakpoints of inversion chromosome In(3LR)Tl^{rv18} and the location of the *Tpi* gene. (B) TPI activity for flies heterozygous for an excision allele and the inversion chromosome In(3LR)Tl^{rv18}. Results indicate some trans-effects still exist, although they are reduced. Dashed red line indicates expected activity for all In(3LR)Tl^{rv18}/Low activity heterozygotes. ** denotes a significant different from the expected value (Tukey's HSD α=0.01, df= 42, k= 6).

2.4 Discussion

The regulation of gene expression has been an area of intense research for nearly as long as we have known about genes. As our understanding of the dynamic nuclear landscape increased, it became clear that a large variety of complex machinery interacts to control the output of genes. The interactions between genetic elements and transcriptional machinery can occur in *cis* (along a chromosome) and *trans* (between chromosomes or between distant regions of a chromosome). One type of *trans*-interaction, found in in *Dipteran* insects, relies on the somatic pairing of

homologous chromosomes. Building on previous work in the *Drosophila melanogaster* model organism, I have created a series of low-activity *Tpi* alleles that are capable of complementing a wild type allele in *trans*. I further identified this interaction as pairing-dependent transvection, as the low activity alleles could not complement a homolog carrying a large inversion. Interactions at *Tpi* are shown to be complex as the up-regulation due to transvection varies significantly between genetic backgrounds. The data from this thesis points heavily toward *Tpi* being an interesting locus for transvection studies, and the small size of the *Tpi* gene will allow for a very targeted analysis of a transvection sensitive allele.

Evidence for transvection at the *Tpi* locus

One of the available screening processes to determine if a genetic locus is permissive to interaction in *trans* is to create heterozygotes which carry a low-activity, or no-activity (knockout), allele and a functional allele (Morris et al. 1998, Lum and Merritt 2011, Bing et al. 2014). In the event that the two homologous regions are regulated independently an additive expression (or phenotype) should be observed. That is to say when a gene is regulated strictly in *cis* an organism heterozygous for a low activity and wild type allele will show approximately half the activity of a homozygous wild type. In the event that the two homologous regions are regulated interdependently, a non-additive expression (or phenotype) should be observed. When the heterozygote exhibits an activity level significantly higher or lower than the additive combination of either homozygote allele investigation into *trans* interactions at that locus is warranted.

When the low activity *Tpi* alleles; *TpiExL1*, *ExL2*, *ExL3*, *ExL4*, are made heterozygous with the wild type allele, *TpiExF*, an activity level above the additive level is observed (Figure 6). This complementation of a wild type allele is very similar to that seen at *Men* (Merritt et al 2005,

2009), although the magnitude of upregulation of the functional copy *Tpi* seems to be lower than at *Men*, however, this apparent difference could be a result of a smaller number of low-activity alleles to sample from. One allele, *TpiExM*, shows a moderate activity relative to the wild type, and when heterozygous with the wild-type *TpiExF* TPI activity levels are additive; no upregulation through transvection is apparent. *TpiExM* likely has a small lesion in the enhancer region, but the promoter is intact and *cis*-preference is preventing complementation. The excision may have damaged the promoter slightly causing the decreased expression and activity relative to the perfect excision *TpiExF*. In the event that the promoter has been damaged and a small portion of the regulatory region was removed, the insulator may have been moved close enough to the promoter to physically prevent the formation of more complex transcription complexes, resulting in decreased expression.

Previous work at *Men* showed that genetic background has a significant influence on upregulation due to transvection (Lum and Merritt 2011, Bing et al 2014). Results at *Tpi* are consistent with findings at *Men* although differences between alleles are, as stated, smaller and less consistent between excision alleles (Figure 7). While there were significant differences between genetic backgrounds in most alleles, two alleles did not show this variation (*TpiExF* and *TpiExL1*). The wild type allele *TpiExF* was not expected to vary significantly with genetic background due to being regulated in *cis*, which is consistent with findings at *Men* (Lum and Merritt 2011, Bing et al. 2014). The lack of variation seen in the *TpiExL1* allele however, is unexpected since regulation in *trans* has been shown to be sensitive to both genetic background and environment at *Men* (Bing et al. 2014). This stability across genetic backgrounds may be due to *TpiExL1* heterozygotes being at near maximum transcript levels regardless of background, q-PCR analysis of heterozygotes can be used to confirm this. Another unexpected result from the

genetic background experiments is the significant difference in TPI activity seen when *TpiExM* is in different genetic backgrounds since the *TpiExM* allele is thought to be regulated in *cis*. The consistently slightly higher than expected values of *TpiExM* heterozygotes, as well as the sensitivity to genetic background, may be due to *TpiExM* being damaged enough to prevent *cis*-preference, while being intact enough to provide moderate transcriptional activity. In order to understand the basis of these unexpected variations a more thorough examination of the regulatory region at *Tpi* is needed to determine the number of different enhancer regions present at this locus, as well as how many of these regions are intact in the experimental excision alleles. The less pronounced transvection effects at *Tpi* may reflect a more rigid regulation of transvection at *Tpi*, possibly a function of the smaller regulatory region. The presence of a class I insulator so close to the promoter could represent a barrier to any regulatory elements from elsewhere in the genome, leaving only the very short regulatory region to control transcription in *cis*, or complement a homologous allele in *trans*.

Ability of inversions to reduce *trans*-activity

Pairing of alleles with chromosomal rearrangements is the classically established method of disrupting somatic pairing between homologous chromosomes used to demonstrate that the complementation seen at a locus is dependent on the pairing of homologous chromosomes. Similar to previous work in the Merritt lab (Bing et al 2014), I used inversion chromosomes to rearrange the genomic architecture and potentially disrupt pairing (Figure 8B, 9B). Results at *Men* showed that a large inversion with breakpoints on either side of the centromere was capable of eliminating transvection based up-regulation (Bing et al 2014). Results at *Tpi* are consistent with those at *Men*;the large inversion which disrupted transvection at *Men* was also capable of disrupting transvection at *Tpi*. Interestingly, another inversion, which has breakpoints on either

side of the centromere, could not completely disrupt transvection at *Tpi*. This incomplete disruption of transvection also seemed to instigate *trans* effects from the putatively *cis* regulated *TpiExM* allele, which appears to behave differently depending on the genetic setting in which it is placed. The effects of the second inversion are somewhat surprising, given that the 3' breakpoint of this inversion is much closer to the *Tpi* locus, and lies within the critical region between the centromere and the *Tpi* gene. Simply looking at the breakpoints, we would have predicted that the second inversion, but possibly not the first, would disrupt pairing and eliminate transvection effects. The fact that we see the opposite pattern underscores the complexity of critical regions for pairing. Our results suggest that the critical region for the *Tpi* locus is somewhere between the centromere and the middle of chromosome arm 3R. This critical region has a limit however, as if an inversion is sufficiently large that it relocates the critical region as well as a large downstream region pairing seems to be partially re-established. The location of *Tpi* at the very distal end of 3R and the common critical region of pairing between *Men*, *Tpi*, and *Ubx* (Bing et al. 2014, Lewis 1954) suggests that inversion chromosome In(3LR)LD6 disrupts the pairing of the entire 3R chromosome arm. This model does not, however, explain why inversion chromosome In(3LR)Tl^{rv18}, which relocates a larger portion of 3R, does not also disrupt pairing fully, but rather reduces the up-regulation attributed to transvection. Further research into visualizing the pairing of homologous chromosomes through Fluorescence In Situ Hybridization (FISH) (Joyce et al 2012) in the presence of inversion chromosomes could explain these conflicting results. Probing for various regions of 3R would allow for visual confirmation that these rearrangements are disrupting pairing rather than introducing regulatory elements which are acting as repressors in *trans* at the *Tpi* locus.

General conclusions

Throughout this study we have generated and characterized a suite of *Tpi* excision alleles as well as established that *Tpi* is a transvection sensitive locus. The generation of gene loci at which to study transvection lays the foundation for further research comparing and contrasting this complementation in a number of unique genomic contexts. The *Triose-phosphate isomerase* gene appears to be permissive to transvection when heterozygous for a wild-type and low activity allele. Further research into complementation at this locus will help create a model for how transvection at this locus is happening as well as shed light on the general characteristics of transvection. The ever expanding number of examples of non-homologous *trans* interactions only helps to underscore the importance of understanding how *trans*-interaction between homologous alleles are possible as the homology of regulatory and coding regions helps simplify the number of contributing factors. Continuing research into all forms of *trans*-interactions may help yield a more accurate central dogma, which allows for a more consistent predictable theory of genetic regulation.

Chapter 3 Future Directions and General Conclusions

3.1 Possible model for transvection at *Tpi*

The results of the heterozygote complementation and inversion heterozygote experiments presented in this thesis can be used to propose a model of regulation in *trans* at *Tpi*. A number of conclusions can be drawn from the genetic architecture around the *Tpi* gene. Specifically the high gene density and presence of an insulator just upstream should restrict accessibility of regulatory elements to the promoter region, preventing assembly of the more complex and variable multi-unit transcription complexes. At the *white* locus, transvection is regulated by the aggregation of multiple proteins, but the relatively small *Tpi* regulatory region suggests that transvection at this locus may involve a different mechanism functions at *Tpi*. The presence of functional genes on either side of the *Tpi* gene region along with a class I insulator located just upstream of the enhancer region can tentatively rule out the looping out of an insulator in *cis* (since the enhancer region is between the insulator and the promoter). The insulator was identified in a metagenomics project using prediction algorithms (Negre et al. 2010), so experimental confirmation of functionality is required before fully dismissing the hypothesis that looping out in *cis* drives the up-regulation we are seeing at *Tpi*. Considering all the evidence, the most likely model for transvection at *Tpi* is a sharing of enhancer elements in *trans* where the enhancer elements associated with the damaged promoter act in *trans* on the homologous intact promoter. In order to confirm this hypothesis, a complementation experiment similar to those performed at the *yellow* locus is required (Morris et al 1998). A series of alleles would need to be generated which keep the promoter at *Tpi* intact but completely removes the *cis* regulatory region. These regulatory deficient alleles would be paired to the promoter deficient alleles generated in this thesis, and assayed for TPI activity. If the hypothesis of enhancer action in *trans*

is correct, these two alleles will complement, showing a non-additive activity profile as the regulatory region of one allele drives the promoter on the homologous chromosome. This complementation could then be tested for resilience against chromosomal rearrangements.

3.2 Creation of more *Tpi* experimental alleles

A larger suite of lesions at the *Tpi* locus would likely give a more complete understanding of the mechanisms of transvection at *Tpi*. I used *P*-element excision-based mutagenesis to create the lesions in this thesis. This method uses imperfect excision of a transposon to remove genetic material surrounding the insertion site of the transposon. Deletion of the genomic region around the P-element site during P-element excision is essentially random, so this method requires collection and screening of a many hundreds of fly lines. The method, does, however target the insertion site, and thus the excision site, making *P*-element mutagenesis a popular alternative to X-ray or ethyl methanesulfonate (EMS) mutagenesis, which are completely untargeted. Currently, there is much interest in even more targeted forms of mutagenesis. Recent advances in our understanding of the immune response of *Streptococcus pyogenes* yielded the discovery of clustered, regularly interspaced, short palindromic repeat (CRISPR) system of genome manipulation (reviewed in Sander and Joung 2014). The CRISPR system uses short RNA sequences called guide RNA (gRNA) to specifically direct mutagenesis at a single locus. This directed mutagenesis is carried out by the CRISPR associated protein 9 (Cas9) protein which comes from *S. pyogenes*. The CRISPR-Cas9 system is capable of introducing mutations approximately 15bp in length, this includes excisions and insertions, although larger lesions are possible if a stepwise approach is taken. The notable advantage of using the CRISPR-Cas9 system is the target specificity as well as the reduction in the amount of labour hours devoted to the creation of mutants. This specificity would allow for the creation of a group of excision

alleles whose promoter or enhancer regions can be disrupted with minimal damage to surrounding genetic material. An additional benefit is the lack of artefacts left behind by the process, which solves the issue of leftover *P*-element fragments containing confounding regulatory elements. A reasonable next step in studying transvection at *Tpi* would be the use of CRISPR-Cas9 mutagenesis to create a series of promoter deficient and enhancer deficient alleles; a series of approximately 20 different alleles which eliminate 15bp of the regulatory region in a stepwise manner would allow us to narrow down precisely where the *trans* acting elements are located. Since we have yet to rule out the possibility that the increased activity we are seeing is from the wild type allele activating the damaged allele, an introduction of a marker to determine where allele transcripts are coming from could help shed some light on the mechanisms of up-regulation at *Tpi*. The inclusion of the regulatory region within the exonic regions of the *Tpi* gene makes this locus a better candidate for this kind of CRISR-based approach than the *Men* locus, which includes potentially 20kb of regulatory sequences.

3.3 Creation of novel inversion chromosomes using the FLP recombinase

The importance of chromosomal inversions in the disruption of chromosomal pairing makes them a critical part of transvection research, but there are a surprisingly small number of inversion chromosomes available. Additionally, these few inversion chromosomes have poorly defined breakpoints, often several kilobases wide. The relationship between inversion chromosomes and pairing disruption has been shown to be complex (reviewed in Duncan 2002, Bing et al. 2014, and this thesis) warranting further research into precisely how inversion chromosomes are able to disrupt pairing. In the interest of understanding pairing dynamics we intended to create inversions with clearly defined breakpoints using the FLP-FLP recombination target (FLP-FRT) recombination system pioneered by Golic and Golic (Golic and Golic 1996a).

The FLP-FRT system uses the FLP recombinase protein isolated from yeast, which interacts strongly with FRT sites in a direction specific manner (Golic and Lindquist 1989). The 5' to 3' orientation of the FRT elements being recombined direct the results of recombinase activity (excision versus inversion). If the two FRT elements are in the same orientation, the FLP recombinase will excise the genetic material between them, but if the FRT sites are in opposite orientations the intervening genetic material will be inverted (Golic and Lindquist 1989, Golic 1991, Golic 1994). The creation of inversions using the FLP-FRT system is outlined in Golic and Golic (1996a). Briefly, a chromosome is made containing two FRT sites in *cis* facing opposite directions. Activation of the FLP recombinase promoter will produce functional FLP protein that will bind the FRT sites and if the two FLP loaded FRT sites come into contact an inversion of the intergenic region will occur (Figure 10). Each of the two FRT sites carries a piece of the *white* reporter gene which will be assembled into a functional copy upon successful inversion, allowing for detection of inversion chromosome bearing flies by their red eyes. The requirement for passive physical interaction of the two FRT sites causes recombination success to decrease as the physical chromosomal distance between the FRT sites increases.

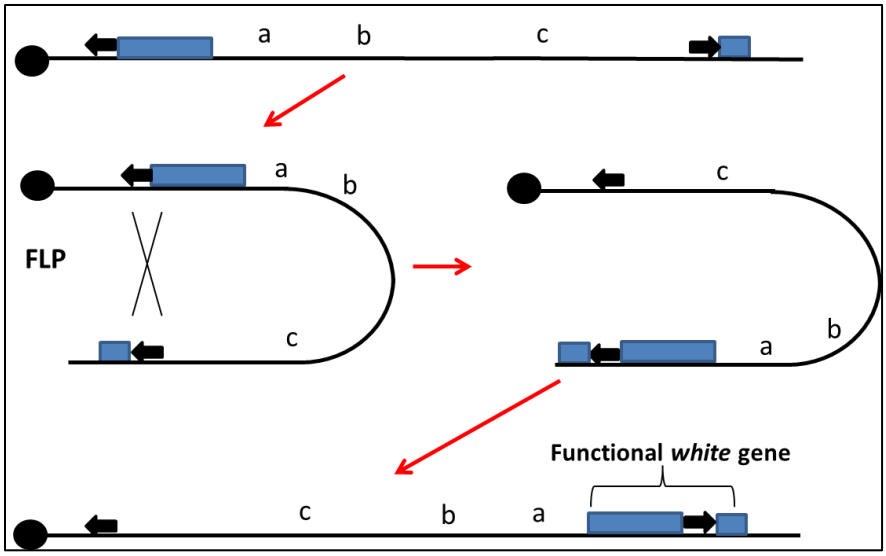

Figure 10 Schematic diagram of the inversion of a piece of a chromosome arm using FLP-FRT mediated recombination. Successful inversion will restore the *white* marker gene allowing for a physical marker for successful inversion.

As part of my thesis research, I attempted to create a suite of inversion chromosomes using the method outlined above. A number of FRT containing Rearrangement Screen (RS) elements were inserted at various sites across the genome by Golic and Golic (1996a) and flies containing these elements are available from the BDSC. The RS elements are constructs containing phenotypic markers as well as FRT sites, allowing for efficient screening of experimental flies. Several RS elements were chosen based on their locations on chromosome arm 3R in order to create an inversion chromosome better suited for investigations at *Tpi*. Figure 11 shows the 15 sites chosen in the effort to create a chromosomal inversion spanning chromosome arm 3R, where an individual fly would have one “RS3” and one “RS5” site in *cis*.

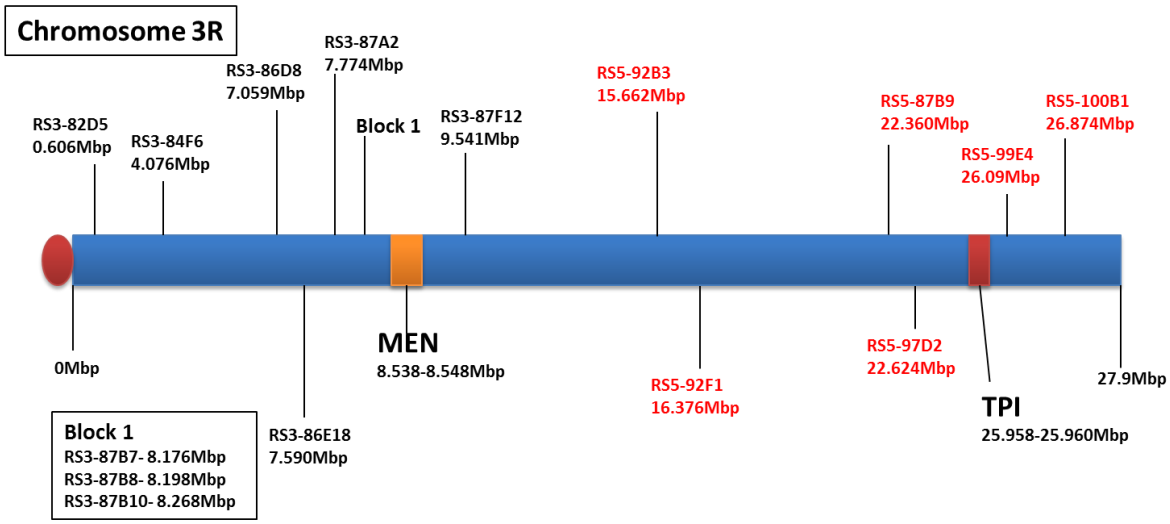

Figure 11 Schematic diagram of the RS insertions used throughout the FLP-FRT inversion project.

After removing several of the RS insertions from consideration due to difficulties in excising internal elements, the remaining 10 (3 RS5 and 7 RS3) were carried forward to recombination experiments. The objective of the recombination experiments was to get one RS3 and one RS5 element facing opposite directions in *cis*. Two successful combinations of RS3/5 in *cis* were recovered and confirmed using PCR. These two recombinant lines were moved forward to the inversion experiments which were performed in 50 individual vials. No inversion chromosomes were recovered from either set of crosses. While I was able to create chromosomes containing two FRT elements in *cis* facing opposite directions I was unable to successfully invert the intervening genetic material. The likely the reason I was unable to recover inversion chromosomes is the distance between my chosen RS sites. The smallest of the two inversions we were trying to create would invert approximately 18 Mbp of chromosome 3R, which is smaller than the largest inversion Golic and Golic (1996a) were able to recover (approximately 28Mbp). The inversion created by Golic and Golic (1996a) was, however, pericentric and their success in

recovering this large an inversion may be due to the heavily tethered centromeric region, a condition my inversions would have lacked.

The recovery rate for inversions of such large sizes was, however, very low, with inversions over 20Mbp occurring successfully in ~0.03% of screened flies (Golic and Golic 1996a) and it is possible that screening of even more lines would allow recovery of the necessary inversions. The successful creation of such large inversions by Golic and Golic (1996a) suggests that it may be possible to successfully invert the region of 3R bracketed by our chosen RS sites, but the low recombination frequency will require a large-scale attempt. It may be possible to artificially increase the chance of the two FRT sites coming into contact by including a *minute* mutation in the flies which has been shown to increase cell cycle times and allow for more time in which chromosomes can interact (Golic and Golic 1996b). Otherwise, a stepwise approach to creation of large inversions is recommended, as shorter range interactions have a much higher frequency of occurrence, although it should be noted a stepwise process would leave a number of artifacts from the insertion and manipulation of the series of RS elements as the process of chromosomal inversion leaves a portion of the RS element at the insertion site.

3.4 Fluorescence In Situ Hybridization analysis of Pairing

The relationship between chromosomal rearrangements and disruption of pairing is complex, as demonstrated in this thesis. A thorough examination of this relationship has yet to be undertaken despite the importance of pairing disruption in transvection research. FISH would allow us to directly observe paired chromosomes and to develop our understanding of pairing dynamics in rearrangement heterozygotes. FISH has been used for a number of years to label and track chromosomal movements and functions by binding fluorescent probes to specific DNA or RNA sequences (Reviewed in Levsky and Singer 2003). While recent advances in probes and image

analysis algorithms have improved to the point that a region as small as tens of kilobases may be visualized and color coded (Beliveau et al. 2012), resolution at that distance is not necessary for large scale analysis of pairing dynamics. Instead probes should be designed to cover several regions of a chromosome arm such that regions near the centromere, the middle of the arm, and the telomere are labelled. These labels would bind separately to the distinct targets allowing each region to be visualized. Paired chromosomes would give a single, overlapping region of label, while unpaired chromosomes would result in multiple regions. It should be noted that each probe should be a different color depending on the chromosome region being targeted, or else each probe will have to be administered individually to prevent false positives for pairing disruption when two signals are seen. One of the primary issues with this technique would be probe design as inversions would cause the binding sites for these probes to be fairly distal to the homologous sites on the other chromosome, this can be overcome by selecting probes which keep inversion heterozygosity in mind. Probes that fall outside of the inversion breakpoints will not share this problem, and careful selection of probed regions can help overcome any issues caused by their relocation of probed regions. The primary purpose of this experiment would be to gain a better understanding of the relationship between rearrangement breakpoints and disruption of pairing, with the added benefit of providing visual evidence that pairing is or is not disrupted in inversion heterozygotes.

3.5 Conclusions

Throughout this thesis I have described the creation of a series of *Tpi* excision alleles whose activity varies from low through to full wild type when homozygous. I have also shown that when heterozygous for both a low-activity and full-activity allele a greater than expected level of transcription is observed, which can be attributed to *trans*-activity between the two alleles. This

trans-activity was then found to be pairing dependant, although the nature of the pairing interaction between alleles is complex. Further investigation into this pairing dependence is needed, including FISH experiments to visualize the degree of pairing disruption caused by rearrangement heterozygosity. The results of the experiments in this thesis have provided the foundation for the model of regulation in *trans* through transvection at *Tpi*, implicating several transcription factors as drivers of *trans*-action. Further investigation into the transcription factors recruited at *Tpi* will develop our understanding of *trans* action at other loci and contribute to a more generalized model for the search and understanding of transvection sensitive loci.

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